

Decreased Toxicity of *Bacillus thuringiensis* subsp. *israelensis* to Mosquito Larvae after Contact with Leaf Litter

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Bacillus thuringiensis subsp. *israelensis* is a bacterium producing crystals containing Cry and Cyt proteins, which are toxic for mosquito larvae. Nothing is known about the interaction between crystal toxins and decaying leaf litter, which is a major component of several mosquito breeding sites and represents an important food source. In the present work, we investigated the behavior of *B. thuringiensis* subsp. *israelensis* toxic crystals sprayed on leaf litter. In the presence of leaf litter, a 60% decrease in the amount of Cyt toxin detectable by immunology (enzyme-linked immunosorbent assays [ELISAs]) was observed, while the respective proportions of Cry toxins were not affected. The toxicity of Cry toxins toward *Aedes aegypti* larvae was not affected by leaf litter, while the synergistic effect of Cyt toxins on all *B. thuringiensis* subsp. *israelensis* Cry toxins was decreased by about 20% when mixed with leaf litter. The toxicity of two commercial *B. thuringiensis* subsp. *israelensis* strains (VectoBac WG and VectoBac 12AS) and a laboratory-produced *B. thuringiensis* subsp. *israelensis* strain decreased by about 70% when mixed with leaf litter. Taken together, these results suggest that Cyt toxins interact with leaf litter, resulting in a decreased toxicity of *B. thuringiensis* subsp. *israelensis* in litter-rich environments and thereby dramatically reducing the efficiency of mosquitocidal treatments.

The bioinsecticide *Bacillus thuringiensis* subsp. *israelensis* is increasingly used worldwide for mosquito control as an environmentally safe alternative to chemicals. Its toxicity to mosquito larvae is due to a composite crystal produced during the sporulation of the bacterium *Bacillus thuringiensis* subsp. *israelensis* (23). This toxic crystal contains four major toxins (Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa) assembled in three different inclusion types held together by a lamellar envelope (13). Cry toxins are able to spontaneously form spherical (Cry4Aa and Cry4Ba) or rhomboidal (Cry11Aa) crystals, while the crystallization of Cyt toxins requires the presence of accessory proteins (35). Commercial *B. thuringiensis* subsp. *israelensis* formulations are mixtures of cells, spores, and parasporal crystals that are sprayed in mosquito breeding sites. After ingestion by mosquito larvae, crystals are solubilized in the alkaline pH of the gut and soluble toxins are activated by gut enzymes. Cry toxins then bind to specific membrane receptors, oligomerize, and perforate the intestinal membrane, leading to larval death, while Cyt toxins do not need receptors to perforate the membrane (4, 5).

Despite an intensive use of *B. thuringiensis* subsp. *israelensis* for years, no firm case of resistance has been described in the field (23, 25). This is imputed to Cyt toxins, which are known to synergize Cry toxin activity, leading to drastically decreased resistance development (4, 46). Cyt toxins are also able to synergize and overcome resistance to other insecticides, such as *Bacillus sphaericus*, in *Culex quinquefasciatus* (43, 47). Developments of new insecticides aim to combine toxins from *B. thuringiensis* subsp. *israelensis*, *B. sphaericus*, and *B. thuringiensis* subsp. *jegathesan* to obtain more-toxic bioinsecticides (11, 28). Cyt toxins are key proteins in these recombinants, promising to enable a long-term use of these new insecticides by decreasing the possibility of resistance development in field mosquito populations (12, 28). Even if the precise mechanism of synergism remains mostly unknown, it has been suggested that Cyt toxins enhance Cry toxin oligomerization and fixation to the intestinal membrane, notably by acting as a “recep-

tor” after its nonspecific insertion into the intestinal membrane (7, 31, 32).

B. thuringiensis subsp. *israelensis* is often said to have low persistence in the field because of a rapid decrease of toxicity and of the amount of spores recovered a few weeks after spraying (17, 18). This lack of residual activity is due mainly to the low stability of *B. thuringiensis* subsp. *israelensis* toxins under field conditions, notably in organic enriched habitats and when exposed to UV light, heat, changes in pH, or bacterial degradation (21, 23). Recently, an increasing number of studies have shown that *B. thuringiensis* subsp. *israelensis* can persist and even proliferate in the field (10, 36, 38). In the laboratory, the selection of *Aedes aegypti* larvae with field-persistent *B. thuringiensis* subsp. *israelensis* present in decaying leaf litter induced multiple resistances to *B. thuringiensis* subsp. *israelensis* Cry toxins, suggesting a differential persistence of the toxins (29). To our knowledge, there is no study on the behavior of *B. thuringiensis* subsp. *israelensis* toxins in leaf litter, which is one of the main constituents of several mosquito breeding sites and mosquitos' main food source. Some publications showed that activated *B. thuringiensis* subsp. *israelensis* toxins can rapidly be adsorbed on clay (in a few hours), which increases their persistence and conserves their toxicity to mosquito larvae (16, 24). The persistence in soil of Cry toxins produced by other *B. thuringiensis* strains active against lepidopteran and coleopteran larvae is also well documented, with the *B. thuringiensis* Cry toxins exhibiting the same pattern of rapid adsorption as the

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B. thuringiensis subsp. *israelensis* Cry toxins (19, 20). Nevertheless, all these studies have been performed on solubilized and activated toxins. In contrast, *B. thuringiensis* subsp. *israelensis*, and also some formulations of other *B. thuringiensis* subspecies, are sprayed in the field as a mixture of crystals and spores. To our knowledge, only one study has evaluated the persistence of crystal Cry toxins sprayed with commercial *B. thuringiensis* subsp. *kurstaki*, which is used to protect cork oak stands in Sardinia (Italy): the toxin can persist up to 28 months after being sprayed into soils (40). Nothing is known about the behavior in the field of the four main *B. thuringiensis* subsp. *israelensis* toxins in their crystal forms.

Here we characterized the behavior of individual and composite *B. thuringiensis* subsp. *israelensis* toxin crystals in water alone and in contact with leaf litter during 7 days. We show that the Cyt toxin amount detectable rapidly decreases (by 60%) when in contact with leaf litter and that its synergistic effect on each individual Cry toxin also decreases (by about 20%). Furthermore, *B. thuringiensis* subsp. *israelensis* composite crystals show a decrease in toxicity by about 70% when in contact with leaf litter. All these results shed light on differential *B. thuringiensis* subsp. *israelensis* toxin persistence in the field and suggest that *B. thuringiensis* subsp. *israelensis* treatment efficiency is strongly related to environmental characteristics of the mosquito breeding sites.

MATERIALS AND METHODS

Mosquito strains. An *Aedes aegypti* Bora-Bora laboratory strain, susceptible to all insecticides, was used for the bioassays. Larvae were reared in tap water and fed with standard amounts of larval food in standard insectary conditions (27°C, 14-h-light/10-h-dark periods, 80% relative humidity).

Bacterial strains and toxin production. Protoxins were produced separately using a crystal-negative strain of *Bacillus thuringiensis* subsp. *israelensis* (4Q2-81) transformed with the plasmids pHT606, pHT618, pWF53, and pWF45 for Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa, respectively (9, 30, 48). These strains were obtained from the Pasteur Institute (Paris, France) or from B. Federici (University of California, Riverside). Spores and crystal suspensions were produced as previously described (29) and conserved in water at -20°C until use. Toxins were quantified on SDS-PAGE using bovine serum albumin (BSA) as a standard. The intensity of each band was estimated, and toxin concentrations were calculated using ImageJ software v.1.41o (15). The same protocol was used to produce wild-type *B. thuringiensis* subsp. *israelensis* inclusion bodies after having isolated spores from the commercial *B. thuringiensis* subsp. *israelensis* VectoBac WG strain in order to remove the commercial formulation effect.

***B. thuringiensis* subsp. *israelensis* extraction and detection by ELISA.** Crystal toxins were solubilized using an alkaline buffer (pH 10.8) containing Na₂CO₃ (50 mM) and dithiothreitol (DTT) (10 mM) and were incubated at 60°C for 1 h. Solubilized toxins were then purified using the ion-exchange column HiPrep 16/10 DEAE FF (Amersham Biosciences) and desalted using the HiPrep 26/10 desalting column (Amersham Biosciences). Toxins were lyophilized and conserved at -20°C until use. Before use, lyophilized toxins were resuspended into distilled water and toxin concentrations were determined by a Bradford assay using BSA as a standard (3). Two aliquots were then used in duplicate as standards for each enzyme-linked immunosorbent assay (ELISA) at concentrations ranging from 2 to 500 ng/ml.

Leaf litters were collected in mosquito breeding sites never treated with *B. thuringiensis* subsp. *israelensis* in the French Rhône-Alpes region. They were constituted of leaves mainly of *Quercus* sp. and some of *Alnus glutinosa*, and their pH was nearly neutral (6.8). The litters were contaminated with commercial *B. thuringiensis* subsp. *israelensis* (VectoBac WG,

3,500 international toxic units [ITU] · mg⁻¹) and left to dry overnight at room temperature in order to be able to powder them, a necessary step for subsequent reproducible toxin extraction. *B. thuringiensis* subsp. *israelensis* toxins were then extracted using the extraction procedure described in French patent no. FR1160365. This extraction procedure is highly reproducible for each toxin (see Fig. S1 in the supplemental material) and for a large range of concentrations, suggesting that the extraction procedure in itself has no impact on the integrity of the toxins. The detection of toxins was performed by sandwich ELISAs using anti-Cry4 (Cry4Aa and Cry4Ba), anti-Cry11 (Cry11Aa), and anti-Cyt (Cyt1Aa) antibodies as described in reference 27, with slight modifications. Goat anti-mouse antibodies conjugated with horseradish peroxidase (CliniSciences) were used to detect anti-toxin antibodies. One hundred microliters of Ultra-TMB (3,3',5,5'-tetramethylbenzidine) (Thermo Scientific) was added, and the reaction was stopped by adding sulfuric acid 2N (H₂SO₄) after 3 min (Cry4) or 5 min (Cry11 and Cyt) of the reaction. Optical densities were measured at 450 nm.

To calculate the amounts of the toxins that bound to the litter, their initial proportions in commercial *B. thuringiensis* subsp. *israelensis* were estimated by solubilizing commercial *B. thuringiensis* subsp. *israelensis* with the same alkaline buffer as the one described above and using it on the same ELISA. The proportion of each toxin in the commercial *B. thuringiensis* subsp. *israelensis* strain was also determined by SDS-PAGE by measuring the intensity of each band using ImageJ software v.1.41o (15). The intensity of bands stained with Coomassie blue is proportional to the size of the molecule. To have unbiased intensity values per toxin, we divided the intensities measured on the gel by the sizes of the corresponding toxins, i.e., 130 kDa for Cry4Aa and Cry4Ba, 70 kDa for Cry11Aa, and 28 kDa for Cyt1Aa (23), before calculating the proportion of each toxin among Cry toxins and among all *B. thuringiensis* subsp. *israelensis* toxins. As the Cry4Aa and Cry4Ba toxins have very similar molecular masses, they cannot be easily distinguished on the gels and were grouped together for proportion estimations.

Effect of litter on Cry toxins and *B. thuringiensis* subsp. *israelensis* toxicity to mosquito larvae. Concentrations of crystal Cry4Aa, Cry4Ba, and Cry11Aa were chosen in order to obtain about 70% larval mortality after 24 h (5.20, 3.13, and 0.97 µg/ml, respectively). Bioassays were performed for nine replicates on 20 third-instar larvae in 50 ml of tap water in plastic cups according to the standard bioassay procedure described by the World Health Organization (41). For each Cry toxin, either the toxin was added to a plastic cup already containing 50 mg of noncontaminated powdered litter (Fig. 1A, unbound toxin) or 50 mg of powdered litter contaminated as previously described (left to dry 3 h and powdered; bound toxin) was added (Fig. 1B). The same protocol was used for two commercial *B. thuringiensis* subsp. *israelensis* mixtures: the dry-powder-formulated VectoBac WG strain (319 ng of formulated product per ml) and the liquid-formulated VectoBac 12AS strain (6.4 × 10⁻⁴ µl/ml). To test for the effect of the insecticide formulation, we also tested a nonformulated *B. thuringiensis* subsp. *israelensis* strain produced in the laboratory using spores isolated from VectoBac (1.7 ng/ml) as previously described (29). Larval mortality was monitored after 24 h. The effect of treatment (contaminated versus noncontaminated) on larval mortality was tested by a linear model performed with R 2.8.1 software (33).

Effect of litter on the Cyt synergistic effect on Cry toxins. Taken separately, Cyt toxins are only a little toxic for mosquitoes, but they are powerful synergists of Cry toxicity. The most convenient way to measure the impact of litter on Cyt1Aa bioavailability was to measure Cyt1Aa's synergistic effect on Cry4Aa, Cry4Ba, and Cry11Aa instead of its direct toxicity to mosquito larvae. The concentration of each Cry toxin was chosen in order to have less than 10% larval mortality after 24 h (585.74, 47.01, and 22.75 ng/ml for Cry4Aa, Cry4Ba, and Cry11Aa, respectively). The concentration of Cyt toxin added to each Cry toxin was chosen in order to obtain about 70% mortality after 24 h (4.23 ng/ml for synergy with Cry4Aa and 21.15 ng/ml for synergy with Cry4Ba and Cry11Aa). Bioassays were performed for nine replicates on 20 larvae in 50 ml of tap

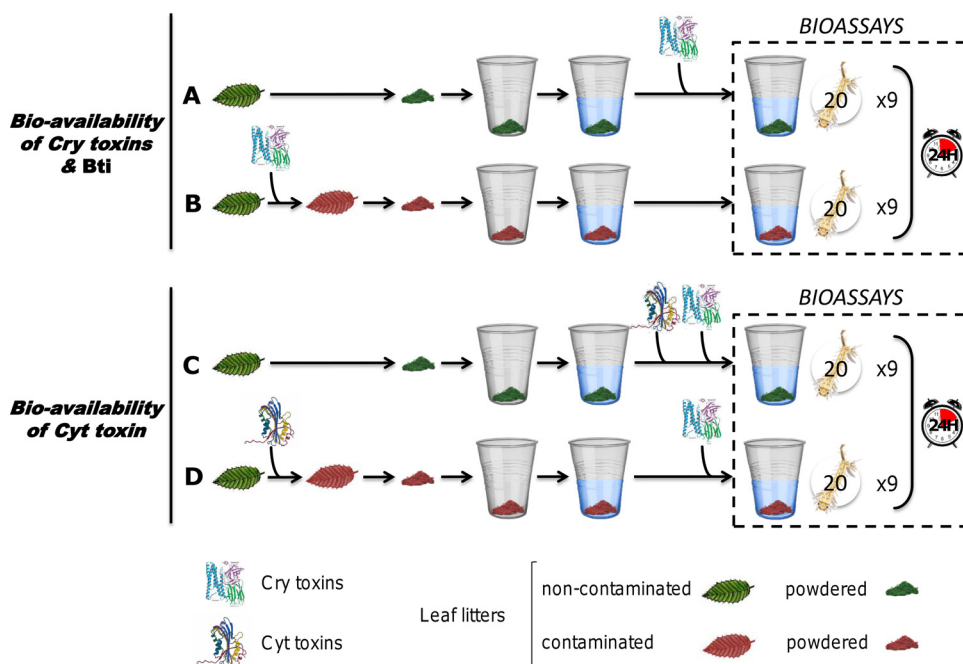


FIG 1 Illustration of the experimental design for the measurement of the effect of leaf litter on the bioavailability of Cry toxins and *B. thuringiensis* subsp. *israelensis* (*Bti*) (A and B) and Cyt toxins (C and D). First protocol: Cry toxins added directly into plastic cups containing noncontaminated powdered leaf litter (A) are compared to Cry-contaminated powdered leaf litter (B) in a bioassay (20 larvae per cup, 9 replicates, mortality monitored after 24 h). The same protocol was followed for commercial and laboratory-produced *B. thuringiensis* subsp. *israelensis* formulations. Second protocol: Cyt and Cry toxins added directly into plastic cups containing noncontaminated leaf litter (C) are compared with Cry toxins added in plastic cups containing Cyt-contaminated leaf litter (D) in a bioassay. The statistical differences between the two conditions (contaminated versus noncontaminated) for each toxin (represented in the same dotted square) were tested after 24 h of larval exposure using a linear model (Cry toxins) or a generalized linear model (*B. thuringiensis* subsp. *israelensis* and Cyt toxins).

water in plastic cups. In this test, the Cyt1Aa toxin was added first either directly into a plastic cup containing 50 mg of noncontaminated powdered litter (Fig. 1C) or bound to 50 mg of litter that was contaminated 3 h, 1 day, 2 days, or 7 days before the bioassay and powdered (Fig. 1D). Larval mortality was monitored after 24 h. The effect of treatment (contaminated versus noncontaminated) on larval mortality was tested by a linear model followed by multiple pairwise comparisons of means (Tukey's honestly significant difference test) performed on R 2.8.1 software (33).

Effect of the time on toxin stability and toxicity without litter. To test whether *B. thuringiensis* subsp. *israelensis* toxins progressively solubilize, degrade, and/or lose their toxicity for mosquito larvae without being in contact with leaf litters, we left aliquots of Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa toxins for 7 days at room temperature or at -20°C . The toxicity of the Cry toxins and the synergism of the Cyt1Aa toxin were measured in the presence of litter in a plastic cup as previously described (Fig. 1A and C). Partial solubilization and/or degradation were estimated by centrifuging toxins at $16,000 \times g$ for 15 min and analyzing the content

of the supernatant with SDS-PAGE (presence of bands corresponding to protoxins and/or degraded toxins) and in ELISAs.

RESULTS

Quantification by SDS-PAGE indicated that the Cyt1Aa toxin represents about 80% of the total amount of toxins present in the commercial *B. thuringiensis* subsp. *israelensis* formulation (Table 1). Among Cry toxins, about 85% were Cry11Aa and 15% were Cry4Aa and Cry4Ba toxins. Quantification of toxins in the commercial *B. thuringiensis* subsp. *israelensis* strain by ELISAs showed nearly the same proportions of toxins (Table 1). ELISAs showed that the proportion of Cyt toxins after *B. thuringiensis* subsp. *israelensis* extraction from leaf litters decreased by about 60% compared to that in the commercial *B. thuringiensis* subsp. *israelensis* formulation, while the proportions of Cry4Aa/Cry4Ba and Cry11Aa among Cry toxins remained unchanged (Table 1).

TABLE 1 Proportions of each toxin among all the toxins or among Cry toxins in a *B. thuringiensis* subsp. *israelensis* formulation in contact or not in contact with litter^a

<i>B. thuringiensis</i> subsp. <i>israelensis</i> state and test	Proportion (%) of each toxin:				
	Among all toxins			Among Cry toxins	
	Cry4Aa and Cry4Ba	Cry11Aa	Cyt1Aa	Cry4Aa and Cry4Ba	Cry11Aa
Solubilized; SDS-PAGE	2.9	15.5	81.6	15.7	84.3
Solubilized; ELISA	1.0	8.1	90.9	11.1	88.9
Extracted; ELISA	12.6	61.7	26.7	17.2	82.8

^a The proportion of each toxin was determined by SDS-PAGE or by ELISAs using solubilized commercial *B. thuringiensis* subsp. *israelensis* or after toxin extraction from leaf litter.

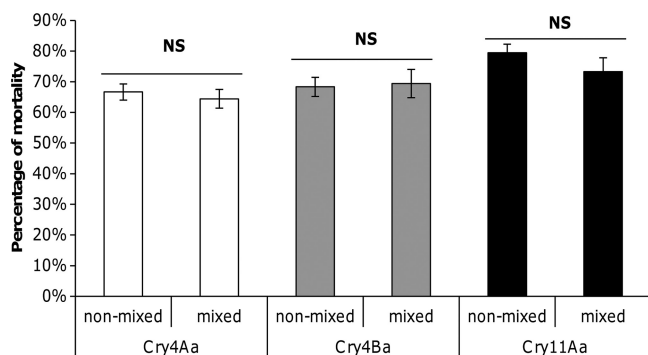


FIG 2 Percentages of larval mortality after exposure to Cry4Aa (white), Cry4Ba (gray), or Cry11Aa (black) mixed or not mixed with leaf litter. Values are the mean percentages and the standard errors from nine replicates per treatment. Nonsignificant differences between bound and unbound toxins for each Cry toxin are indicated as “NS” between the two bars.

Toxicity of Cry toxins to mosquito larvae (Fig. 1, condition A versus condition B) was not affected by their contact with leaf litter (Fig. 2). Our results clearly indicate the synergistic effect of Cyt on Cry toxins. We observed 73%, 61%, and 57% increased larval mortalities, respectively, for Cry4Aa, Cry4Ba, and Cry11Aa when Cyt was added in bioassays. In contrast, when Cyt was put in contact with leaf litter (Fig. 1, condition D versus condition C), significant decreases of the synergistic effects on Cry4Ba and Cry11Aa for all the exposure durations and on Cry4Aa for 1 and 7 days of exposure were observed (Fig. 3A, B, and C). We observed mean decreases of 20.28%, 22.22%, and 17.18% in the synergistic effect of Cyt1Aa on Cry4Aa, Cry4Ba, and Cry11Aa, respectively. Toxicities of the two commercial *B. thuringiensis* subsp. *israelensis* strains, VectoBac WG and VectoBac 12AS, and the laboratory-produced *B. thuringiensis* subsp. *israelensis* strain were significantly reduced by 76%, 78%, and 70%, respectively, when mixed

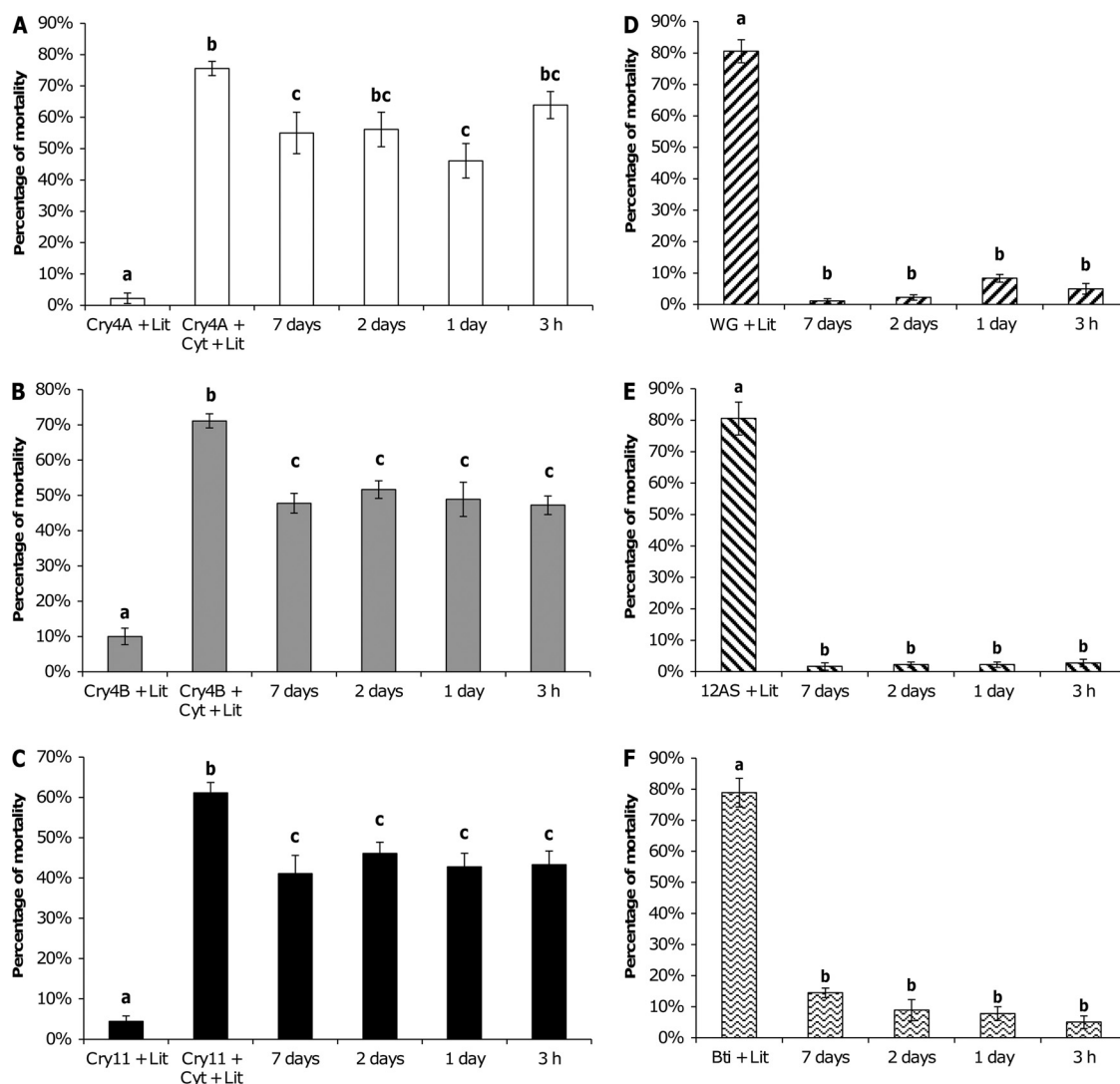


FIG 3 (A to C) Percentages of larval mortality after exposure to Cry4Aa (A), Cry4Ba (B), or Cry11Aa (C) toxins without Cyt1Aa (Cry + Lit) or with Cyt1Aa and not mixed (Cry + Cyt + Lit) or mixed with leaf litter for 3 h, 1 day, 2 days, or 7 days. (D to F) Percentages of larval mortality after exposure to commercial *B. thuringiensis* subsp. *israelensis* VectoBac WG (D), VectoBac 12AS (E), or a *B. thuringiensis* subsp. *israelensis* formulation produced in nutrient agar (F) not mixed (+ Lit) or mixed with leaf litter for 3 h, 1 day, 2 days, or 7 days. Values are the mean percentages and standard errors from nine replicates per treatment. Significant differences ($P < 0.05$) between treatments (contaminated versus noncontaminated) are indicated by different letters above the bars.

with leaf litters (Fig. 1, condition A versus condition B, and Fig. 3D, E, and F). For all the bioassays performed, the duration of toxin exposure to leaf litter did not significantly affect larval toxicity (Fig. 3). Moreover, larval toxicity was unchanged when toxins were left in water at room temperature for 7 days (see Fig. S2 in the supplemental material), and neither solubilization nor degradation was detected by both SDS-PAGE and ELISAs.

DISCUSSION

Cyt toxins are known to enhance Cry toxin activity and to delay the development of *B. thuringiensis* subsp. *israelensis* resistance in mosquitoes (4, 46). *B. thuringiensis* subsp. *israelensis* contact with leaf litter induced an important decrease in Cyt1Aa toxin bioavailability (60%), whereas the Cry4 and Cry11Aa toxins remained unaffected. Cyt toxins seem to have strong and irreversible interactions with litter, limiting desorption during the extraction step. Such an interaction has already been described for solubilized and activated Cry1Aa toxin in contact with different soils, with the amount of extractable *B. thuringiensis* toxin declining rapidly during the first 2 weeks of contact (19). Nevertheless, the context described in our study is far from this example, as we focused on leaf litters and on *B. thuringiensis* subsp. *israelensis* toxins in their crystal form. Further investigations are needed to understand how Cyt toxins can interact so strongly with litters in their crystal form. These results also show that *B. thuringiensis* subsp. *israelensis* must be considered a combination of inclusions with different behaviors in the field and not a cohesive inert crystal.

Bioassays showed that Cry contact with leaf litters did not induce any alteration of their toxicity to *A. aegypti* larvae. Cry toxins are easily extractable and immunologically detectable when mixed with leaf litter, and they conserve their insecticidal properties for mosquito larvae.

In contact with leaf litter, the synergistic effect of Cyt on *B. thuringiensis* subsp. *israelensis* Cry toxins decreased by about 20%, regardless of the contact duration. Cyt toxins seem to bind very quickly to leaf litter, as the 20% loss in synergy occurred after only 3 h of exposure. There is a discrepancy between the proportion of unextractable Cyt toxin from leaf litter (60%) and the decrease of its synergistic effect on Cry toxins (20%). Some mechanisms can be hypothesized to try to explain this phenomenon.

(i) This discrepancy may be due to the high synergy power of Cyt toxins, as only small amounts of Cyt toxin are needed to exponentially increase Cry toxicity to mosquito larvae. Therefore, the small proportion of still-extractable and bioavailable Cyt toxins may be sufficient to induce 80% of the total synergistic effect. Indeed, it was observed that Cyt toxins are able to synergize other toxins over a large range of toxin ratios (47). Furthermore, Cyt toxin activity may be enhanced by litter components ingested by larvae together with protoxins, as tannic acids have already been described to enhance the toxicity of *B. thuringiensis* subsp. *kurstaki* for *Trichoplusia ni* (14). However, the impact of tannic acids is not clear, as they were shown to inhibit the toxicity of *B. thuringiensis* subsp. *aizawai* for *Spodoptera litura* (22).

(ii) A part of the Cyt toxins linked to the litter may still be able to synergize Cry toxins. It has been demonstrated that solubilized Cry toxins bound to soil can conserve their toxicity (16, 24), but it is still unknown whether Cyt toxins have the same behavior.

(iii) Cyt toxins may be degraded more rapidly than Cry toxins by microbes present in the leaf litter. Indeed, *B. thuringiensis* subsp. *israelensis* is known to be susceptible to microbial degrada-

tion (23). However, microbial degradation is expected to increase with exposure time. The fact that we did not observe an effect of exposure time either on the proportion of Cyt detected or on its synergistic effect on Cry toxins rules out the role of microbial degradation in differential *B. thuringiensis* subsp. *israelensis* toxin effects when mixed with leaf litter.

(iv) Bound Cyt toxins may experience three-dimensional (3D) conformational modifications, inducing a reduction of its synergistic activity and/or a reduction of its immunological detectability by ELISA. It has been shown that Cry toxins bound to the soil (37) or to leaf litter (present study) conserve their entire antigenicity and insecticidal activity. However, Cyt toxins have very different 3D conformations than Cry toxins and may interact differently with the heterogenic sorbent surface of the leaf litter, explaining why they partly lose their immunological detectability and synergistic effects (26). Even if the toxin extraction protocol coupled with ELISAs showed good efficiency and reproducibility for all toxins (see Fig. S1 in the supplemental material), it cannot be excluded that the drying and powdering steps may differently affect the toxins' stability, especially for the Cyt1Aa toxin, as proportions of Cry toxins remained the same as in SDS-PAGE.

(v) Contrary to Cry toxins that need receptors to perforate the intestinal membrane (23), Cyt toxins can directly insert into the membrane of epithelial cells due to their tertiary structure (5). This ability might also facilitate the binding of Cyt to litter, although plant epithelial cells have a structure that is very different from that of animal cells, with a rigid cell wall not composed of lipids *a priori* not favoring Cyt insertion. Since the synergistic effect of the Cyt1Aa protein on Cry toxins seems to be its role as a Cry "receptor," the interaction of Cyt proteins with litter might therefore inactivate this receptor role.

In contact with leaf litter, the toxicity of *B. thuringiensis* subsp. *israelensis* decreased by about 70%, regardless of the contact duration. No effect of the commercial formulations was evidenced, as the decreased toxicities were nearly the same for the two commercial *B. thuringiensis* subsp. *israelensis* formulations and for the laboratory-produced *B. thuringiensis* subsp. *israelensis* formulation. The strongly decreased toxicity of the composite *B. thuringiensis* subsp. *israelensis* crystal in contact with leaf litter can be explained by two potential nonexclusive mechanisms. (i) *B. thuringiensis* subsp. *israelensis* crystals contain a lamellar envelope, which maintains together the different inclusion types (13), that is typically absent from individual Cry and Cyt toxin productions. This envelope may interact strongly with leaf litter particles, leading to a major decrease in bioavailability of all the toxins. (ii) As Cyt toxins lost about 20% of their synergistic effect on all *B. thuringiensis* subsp. *israelensis* Cry toxins because of contact with leaf litter, the important decreased bioavailability of the Cyt toxin may affect all Cry toxins contained in the *B. thuringiensis* subsp. *israelensis* formulation. Therefore, the observed effect might simply be due to the cumulative effect of the decreased synergy of Cyt1Aa with each Cry toxin, leading to a severe loss of *B. thuringiensis* subsp. *israelensis* toxicity.

Cyt toxins bound to leaf litter lost about 20% of their synergistic effect on all *B. thuringiensis* subsp. *israelensis* Cry toxins. This might explain why the selection of an *A. aegypti* laboratory strain using decaying leaf litters containing field-persistent *B. thuringiensis* subsp. *israelensis* gave rise after 18 generations to a mosquito strain exhibiting consistent levels of resistance to separate *B. thuringiensis* subsp. *israelensis* Cry toxins (6 to 30 resistance ratios)

but only a moderate resistance to the full commercial *B. thuringiensis* subsp. *israelensis* mixture (29). It was demonstrated that even in the presence of Cyt toxins, resistance to Cry toxins and Bin toxins can evolve in selected mosquito strains (42, 44–46). Therefore, the decreased bioavailability of Cyt toxins because of contact with leaf litter may have facilitated the development of Cry resistance in the case of the *A. aegypti* LiTOX strain. In the field, a long-term exposure of larvae to an altered mixture of persistent Cry toxins with low levels of synergistic Cyt toxins may more easily lead to the evolution of resistance to Cry toxins and, at term, to *B. thuringiensis* subsp. *israelensis*. As increasing numbers of recombinant *B. thuringiensis* strains containing Cyt toxins are developed, our results suggest that their efficiency, essentially based on Cyt synergism, might prove to be much lower in the field than in laboratory conditions. These findings have to be taken into account to manage resistance evolution in mosquito breeding sites treated with *B. thuringiensis* subsp. *israelensis* and to evaluate the efficiency of *B. thuringiensis* subsp. *israelensis* and new *B. thuringiensis* subsp. *israelensis*-based insecticides in the field.

Numerous studies have evaluated *B. thuringiensis* subsp. *israelensis* residual activity in many different conditions, including rivers, lotic zones, benthic zones (1, 2, 39), saltmarshes (8), and water tanks and containers (6, 34). It was shown that this residual activity ranged from a few days to several months, and many environmental factors (e.g., UV light, temperature, etc.) were shown to have an impact on *B. thuringiensis* subsp. *israelensis* residual activity (21, 23), but so far, nothing is known about their action on each *B. thuringiensis* subsp. *israelensis* toxin. To our knowledge, this is the first time one major component of several mosquito breeding sites is shown to have a differential impact on Cyt and Cry toxins, leading to a dramatic loss of *B. thuringiensis* subsp. *israelensis* toxicity. The present work opens new ways to better understand how multiple toxins in their crystal form can interact with leaf litter and why *B. thuringiensis* subsp. *israelensis* quickly loses its toxicity for mosquito larvae in the field. As leaf litters are not present in all the mosquito breeding sites, the same experiments have to be performed with clays to see if this phenomenon is specific to leaf litter or if it can be generalized to other components of mosquito breeding sites. These findings must be taken into account for an integrated use of *B. thuringiensis* subsp. *israelensis* for mosquito-cidal treatments and for adapting the treatment strategies to the composition of the treated areas.

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